CHAPTER 1

Introduction

1.1 Overview

Medical diagnosis is the way that can examine the diseases in hospital laboratory by determination of each disease biomarker in biological samples of the patient such as blood, serum and urine. The higher or lower level of biomarker from the normal range is the indicator of disease.

Immunoglobulin G (IgG) is an important component in the immune system that is recognizing virus and bacteria for living body. They are the biomarker of many diseases such as rheumatoid arthritis, liver diseases, cancers, and infectious diseases. Moreover, they can interact specifically with anti-IgG and use this for diagnosis. Analytical methods have been developed for determination of IgG such as radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay, surface plasmon resonance (SPR), and quartz crystal microbalance (QCM) but these techniques are high cost, using many substrates, and analysis is done in specialized laboratory. Electrochemical immunosensor is an interested technique which is a device includes two parts: bioreceptor which selectively detect of substance of interest such as enzyme, antibody, nucleic acid and DNA and transducer which is the device that can transduce result of reaction or interaction between bioreceptor and substance to electrical signal. Moreover, electrochemical immunosensor has the attractive advantages such as rapidity, portable, low cost, fast signal response and fast analysis. In addition, it can be applied with many materials on an electrode surface. The deposition of biomolecule on the electrode surface was reported using many materials (e.g. gold nanoparticles (AuNPs), graphene oxide (GO) [Ahour, 2016; Chen, 2016; Liu, 2015]) that can provide high stability and sensitivity for the sensors. In the recent year, electrochemical sensors have been reported for determination of IgG that used glassy carbon electrodes (GCEs) or screen-printed carbon electrode (SPCEs) which was deposited by materials or biomolecules that can recognize of IgG. In the process to detect IgG, batch voltammetric analysis techniques such as cyclic voltammetry (CV), square wave voltammetry (SWV), differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS) and amperometry were used but all of these are non automatic technique thus flow analysis for determination of IgG is interested to be developed.

Thus in this thesis, sequential injection analysis with electrochemical immunosensors based on SPCEs modified with GO and covalently attached with anti-HIgG for sensitive determination of human immunoglobulin G (HIgG) was studied.

1.2 Immunoglobulin G

Immunoglobulin (Ig) [Immune Deficiency Foundation, 2013] is antibodies and glycoprotein molecules in blood that are produced by plasma cells (white blood cells). The concentration of HIgG in blood of normal adults is in the range from 9.4 to 15.1 mg mL^{-1}. They act as a critical part of the immune response by specifically recognizing and binding to particular antigens, the structure of IgG as shown in Figure 1.1.

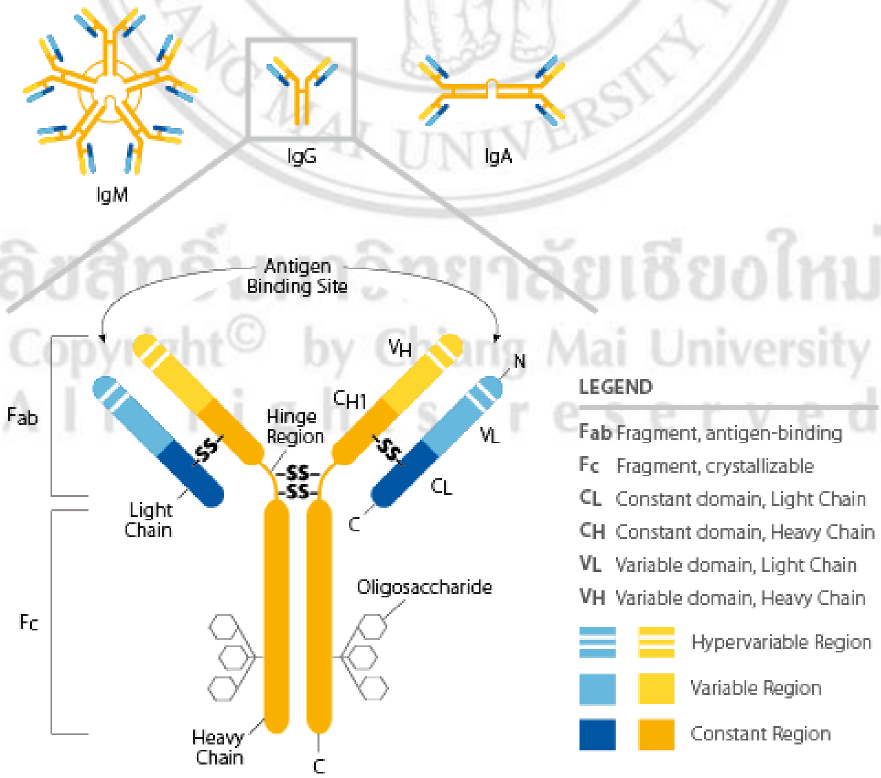

Figure 1.1 The structure of IgG [Affymetrix eBioscience, 2016].

The antibody immune response is highly complex and exceedingly specific. They have five isotypes [Affymetrix eBioscience, 2016] that include IgA, IgD, IgE, IgG and IgM. IgG is the smallest one but most common antibody (75% to 80%) [WebMD, 2015] of all the antibodies in the body. The IgG antibodies are responsible for fighting bacterial and viral infections in the body. They are a potential biomarker of various diseases [Jumpathong, 2016] such as cancers, Alzheimer's disease, inflammatory bowel disease, and autoimmune disease. Therefore, the determination of IgG amount in blood is necessary for medical diagnosis. In this research, we are interested in HIgG. The HIgG antibodies are found in all body fluids. The determination of HIgG has been reported in many methods that using principle of physical, chemical and/or biological properties. In the part of physical properties, there are surface plasmon resonance and quartz crystal microbalance techniques and in the part of chemical or biological properties, there are fluorescence assay, chemiluminescence, and enzyme-linked immunosorbent assay [Zhao, 2007]. However, all of these have long analysis time, having sample pretreatment before analysis, and instruments or reagents are high cost. Electrochemical immunosensor is one of the techniques that was widely studied in the recent year and could effectively determine IgG [Qiu, 2010; Zhang, 2016]. There are many literatures reported the determination of IgG by various techniques as shown in Table 1.1.

Table 1.1 Literature reviews on determination of IgG by various techniques.

Title	**Method**	**Linear range ($ng\ mL^{-1}$)**	**LOD ($ng\ mL^{-1}$)**	**Sample**	**Ref.**
Determination of human IgG by solid substrate room temperature phosphorescence immunoassay based on an antibody labeled with nanoparticles containing Rhodamine 6G luminescent molecules	SS-RTP-IA	0.156 - 50.0	-	Human serum	[Jia-Ming, 2005]
Immunoassay by detecting enhanced resonance light scattering signals of immunocomplex using a common spectrofluorometer	RLS	-	-	Human serum	[Zhao, 2006]
Concentration dependence of IgG–protein A affinity studied by wireless-electrodeless QCM	QCM	0.500 - 100,000	0.500	-	[Ogi, 2007]
Electrochemiluminescence Immunosensor Based on CdSe Nanocomposites	ECL	0.02 - 200	0.001	Human serum	[Jie, 2008]
Ultrasensitive electrochemiluminescence immunosensor based on luminol functionalized gold nanoparticle labeling	ECL	0.0075 - 0.1	0.001	Human serum	[Tian, 2010]

Table 1.1 (Continued)

Title	Method	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	Sample	Ref.
Magnetic nanocomposite of anti-human IgG/COOH-multiwalled carbon nanotubes/ Fe_3O_4 as a platform for electrochemical immunoassay	CV	30 - 1000	25	Human serum	[Zarei, 2012]
Novel SPR biosensors based on metal nanoparticles decorated with graphene for immunoassay	SPR	0.15 - 40.00 g mL^{-1}	-		[Zhang, 2013]
Determination of IgG levels in bulk ewe’s milk	ELISA	-	-	Bulk ewe’s milk	[Galán-Malo, 2014]
Ultrasensitive label-free electro chemiluminescence immunosensor based on N-(4-aminobutyl)-N-ethylisoluminol-functionalized graphene composite	ECL	0.0001 - 10	0.00005	Human serum	[Shu, 2015]
Highly sensitive covalently functionalized light-addressable potentiometric sensor for determination of biomarker	LAPS	0 - 150,000	150	-	[Liang, 2016]

1.3 Electrochemical sensor

Electrochemical sensor is the system that included electrochemical cell (three electrodes: working electrode (WE), auxiliary electrode (AE) and reference electrode (RE)) and processor (potentiostat and computer) called transducer as shown in Figure 1.1. They can detect the changing of reaction or interaction on the electrode surface of working electrode which were modified by biomolecules (bioreceptor) in order to recognize an analyte. In the part of transducer, it was developed for better electrical conductivity, stability and biocompatibility thus nanomaterial, composite, polymer that are useful for binding with bioreceptor were deposited or immobilized on the electrode. Electrochemical sensors based on electrodes modified with many materials were widely developed for determination many analytes as shown in Table 1.2. From the literature reviews, gold seeds decorated on magnetic core Fe_3O_4 nanoparticles (Fe_3O_4@Au) [Samphao, 2015], gold nanoparticle with sulfo group functionalized multi-walled carbon nanotubes (Au@MWCNTs-SO_3H) [Li, 2017], toluidine blue (TB) with cuprous oxide nanowires decorated graphene oxide nanosheets (TB@Cu_2O@GO) nanocomposites [Wang, 2017], overoxidized polypyrole decorate with gold nanoparticle (OPPy-Au_{nano}) [Amouzadeh Tabrizi, 2016], and GO [Jumpathong, 2016] were used to deposite on electrode surface because these materials can bind with biomolecule or bioreceptor by using Au-S bond and hydrogen bond, respectively. It is interesting that these materials actually work, so in this work GO was used for deposition on electrode surface due to the oxygen-containing functional groups in GO render hydrophilicity and it can be covalently to ameliorate GO properties with nanoparticles, organic compounds, bimolecules, and GO offer a high specific surface area and excellent electrochemical properties (electron transfer) [Jumpathong, 2016].

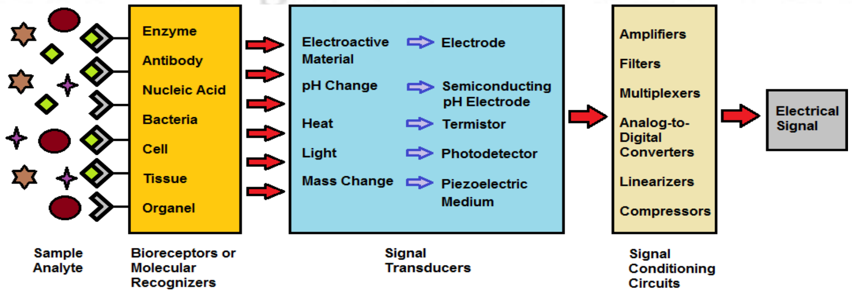

Figure 1.2 Electrochemical sensor [Intechopen, 2015].

Table 1.2 Literature reviews on electrochemical sensor.

Title	**Working electrode**	**Technique**	**Analyte**	**Ref.**
Determination of chromium in estuarine waters by catalytic cathodic stripping voltammetry using a vibrating silver amalgam microwire electrode	SAME	CSV	Chromium	[Espada-Bellido, 2013]
Screen-printed carbon electrodes modified with lead film deposited using different plating methods as sensors in anodic stripping voltammetry	PbF/SPCE	ASV	Zinc	[Tyszczuk-Rotko, 2013]
Sensitive electrochemical immunosensor for α-fetoprotein based on graphene/SnO_2/Au nanocomposite	Anti-AFP/SnO_2/Au/RGO/GCE	DPV	α-fetoprotein (AFP)	[Liu, 2015]
Single-walled carbon nanotubes covalently functionalized with polytyrosine: A new material for the development of NADH-based biosensors	ADH/SWCNT-Polytyr/GCE	Amperometry	Ethanol	[Eguílaz, 2016]
A novel electrochemical biosensor based on Fe_3O_4 nanoparticlespolyvinyl alcohol composite for sensitive detection of glucose	GOx/PVA-Fe_3O_4/Sn	CV	Glucose	[Sanaeifar, 2017]

1.4 Screen-printed carbon electrode

The commonly used working electrode is GCEs and gold electrode (GE) because they have low background current and wide potential window. However, GCEs and GE are high cost and need to be polished with alumina or sonicate to remove materials on electrode surface before use. Therefore, in many researches home-made SPCEs were used instead of GCEs because of its low cost, disposable and having good activity as well as GCEs. For instance, Reanpang et al. [Reanpang, 2015] determined hydrogen peroxide (H_2O_2) by using palladium (Pd) with carbon nanotube (CNT) modified on the home-made SPCE. The result shows detection of H_2O_2 in the range of 0.1-1.0 mM, with detection limit of 20 µM. Moreover, Upan et al. [Upan, 2016] developed a simple SPCE modified with CNT for determination of hydroquinone. The result shows that the proposed system provided a linear range of 1-50 µM with detection limit of 0.1 µM. The developed system provided high stability, good repeatability, high sample throughput, and low reagent consumption. Because of their advantages of SPCEs, thus SPCEs were used as working electrode in this work.

1.5 IgG immunosensor

There are several electrochemical methods that used to determine IgG. For example, Liu et al. [Liu, 2015] fabricated a label-free immunosensor based on 3D-structured reduced graphene oxide-multiwalled carbon nanotube-palladium nanoparticles nanocomposite (rGO-MWCNT-Pd) for detection of HIgG and used CV and SWV to characterize recognizability of HIgG. Zhang et al. [Zhang, 2016] developed a nanohybrid of Ag@Au NRs and Fe_3O_4 nanosphere via the polydopamine (PDA) coating, and then demonstrated a non-enzymatic (anti-IgG and IgG) electrochemical immunosensor using the Ag@AuFe_3O_4 as an efficient nanocatalyst for H_2O_2 reduction. They used CV to characterize the nanomaterial modified electrode toward hydrogen peroxide reduction and used amperometry to determine sensitivity of the immunosensor based on Ag@Au-Fe_3O_4. Amouzadeh Tabrizi et al. [Amouzadeh Tabrizi, 2016] fabricated an electrochemical HIgG immunosensor using overoxidized polypyrrole decorated with gold nanoparticle modified screen printed electrode (OPPy-Au_{nano}/SPE) and used EIS and CV to characterize the assembled process of the immunosensor in $[Fe(CN)_6]^{3-/4-}$. In addition, literatures that developed electrochemical immunosensor for determination of IgG were reported in the Table 1.3. They used immunointeraction between anti-IgG and IgG that immunointeraction was at specific active site of anti-IgG. Although, these methods provided high sensitivity, acceptable selectivity, reproducibility, and stability but they involve many steps of manual operation and high reagents and time consumption. Flow analytical systems can solve these problems and help to improve performance of the immunoassay in terms of more automated, more precise, and less amounts of chemical and time consumption. Amperometry is an electrochemical technique that can use for determination of HIgG. It includes 3 electrodes (WE, RE and AE). The principles of amperometry are fixing the potential that applied to WE with respect to RE (Ag/AgCl) and monitoring the current signal between WE and AE. Current signal is directly related to concentration of analyte. Moreover, this technique has more advantages such as high sensitivity because of hydrodynamic of mass transfer and fast chemical equilibrium. Therefore, development of electrochemical automatic and low reagent consumption method are interested such as sequential injection analysis with amperometry.

Table 1.3 Literature reviews on determination of IgG by electrochemical immunosensor.

Title	Working electrode	Method	Linear range	LOD	Ref.
Development of an immunosensor for the determination of rabbit IgG using streptavidin modified screen-printed carbon electrodes	Anti-RIgG/ streptavidin /SPCE	SWV	5×10^{-11} - 1×10^{-9} M	5×10^{-11} M	[Díaz-González, 2005]
A label-free electrochemical immunoassay for IgG detection based on the electron transfer	Anti-HIgG/PTH-MB/GNP/GE	DPV	10 - 104 ng mL^{-1}	3 ng mL^{-1}	[Qiu, 2010]
Magnetic nanocomposite of anti-human IgG/ COOH-multiwalled carbon nanotubes/Fe_3O_4 as a platform for electrochemical immunoassay	Anti-HIgG/ COOH-MW CNTs-Fe_3O_4/ GE	CV	30 - 1000 ng mL^{-1}	25 ng mL^{-1}	[Zarei, 2012]
A double signal electrochemical human immunoglobulin G immunosensor based on gold nanoparticles-polydopamine functionalized reduced graphene oxide as a sensor platform and AgNPs/carbon nanocomposite as signal probe and catalytic substrate	Ab_1/AuNPs-PDA-rGO/GCE	DPV	0.01 - 100 ng mL^{-1}	0.001 ng mL^{-1}	[Zhang, 2016]

1.6 Sequential injection analysis

Sequential injection analysis (SIA) was developed by Ruzicka and Marshall in 1990 [Ruzicka, 1990] as alternative to flow injection analysis (FIA). Main components of SIA consist of a syringe pump, a selection valve, and a detector as shown in Figure 1.3. Syringe pump is a device which controls mobility of carrier and various reagents. Selection valve is a multi-port valve which the ports are connected to sample and reagent vessels, and also to a detector. Holding coil is the reactor that sample and reagent(s) are mixed and reaction was occurred.

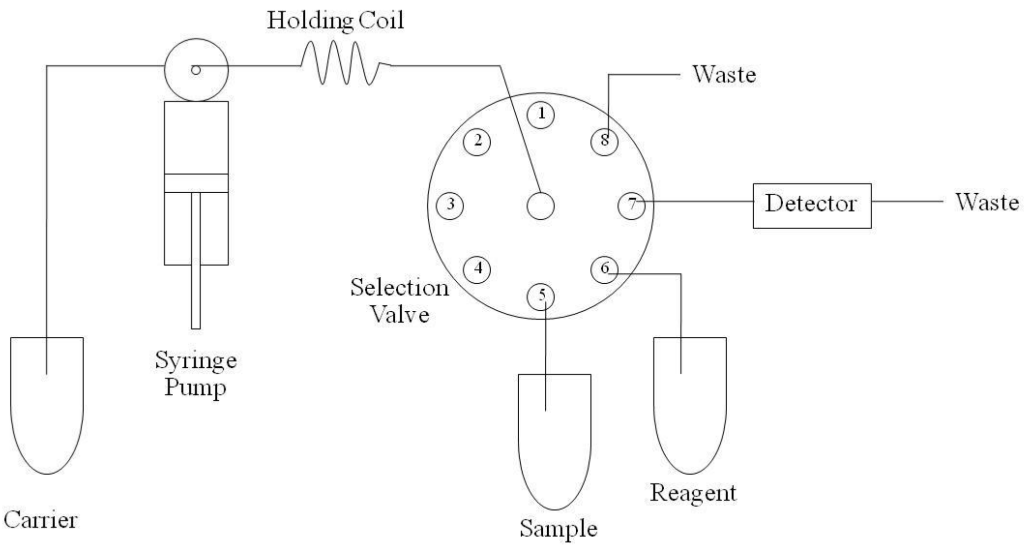

Figure 1.3 SIA system.

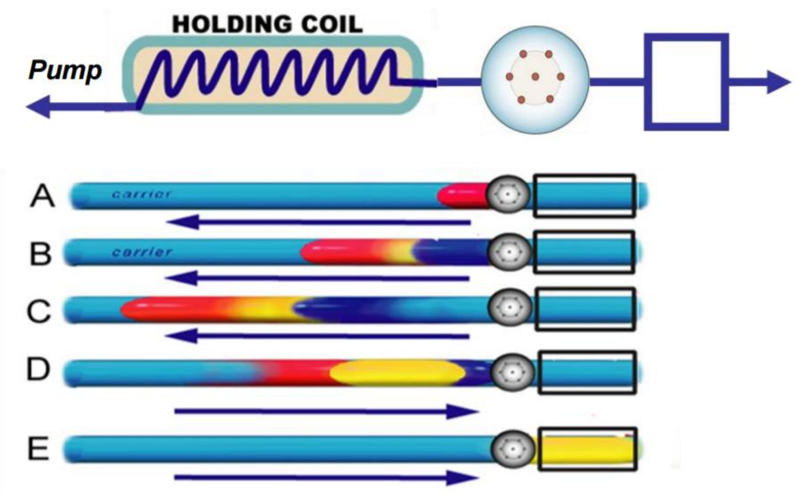

Figure 1.4 The principle of SIA [FIAlab, 2009].

The principle of SIA is illustrated in Figure 1.4. First, the carrier is filled in holding and the reagent and sample are filled in each port. Second, sample (red) is aspirated to a holding coil followed by reagent (blue). Third, sample and reagent are mixed to produce the product as shown in yellow and the carrier is aspirated respectively. Then, the product is dispensed to the detector and the peak height is proportional to the concentration of the analyte. SIA is an automatic method because of computerized control and it uses reagent in µL to mL levels. Furthermore, this method can select various reagents to form reaction respectively and it can apply to electrochemical analysis.

In the recent year, SIA was developed to use with many instruments as shown in Table 1.4. The reported showed SIA could be applied with many methods for more advantages such as providing a good reagent control and using less labor for analysis. However, SIA-amperometry with developed electrode for HIgG determination is never reported, thus this work will use SIA system for dispensing the reagent to the detector unit.

Table 1.4 Literature reviews on application of SIA system.

Title	Detection system	Analyte	Ref.
Sequential injection Fourier transform infrared spectroscopy for the simultaneous determination of organic acids and sugars in soft drinks employing automated solid phase extraction	Fourier transform infrared spectroscopy (FTIR)	Organic acids and sugars	[LeThanh, 2000]
Determination of aluminum by electrothermal atomic absorption spectroscopy in lubricating oils emulsified in a sequential injection analysis system	Electrothermal atomic absorption spectroscopy (ET-AAS)	Aluminium	[Burguera, 2005]
Sequential injection absorption spectrophotometry using a liquid-core waveguide: Determination of p-arsanilic acid in natural waters	Long pathlength absorbance spectrophotometry (LPAS)	*p*-arsanilic acid	[Roerdink, 2005]
Sequential injection analysis with electrochemical detection as a tool for economic and rapid evaluation of total antioxidant capacity	Amperometry	Total antioxidant capacity	[Chan-Eam, 2011]
Fast separation of red colorants in beverages using cyano monolithic column in sequential injection chromatography	Chromatography	Red colorants	[Chocholouš, 2017]

1.7 Indirect determination of IgG

This work will develop SIA system with electrochemical technique to determine HIgG that used SPCE modified with GO as an electrode. However, immunoreaction between anti-HIgG and HIgG did not produce electron that can be detected as electrical current signal by the electrochemical method. Therefore, redox probe such as ferri/ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) was used for signal transduction. In addition, $[Fe(CN)_6]^{3-/4-}$ provided higher sensitivity than other redox probe such as quinine, dopamine, ascorbic acid, and 3,3′,5,5′-tetramethylbenzidine (TMB) when using equal concentration. The indirect determination of IgG by using $[Fe(CN)_6]^{3-/4-}$ was proposed. An initial current signal (I_0) was measured and the decrease current signal (I) was monitored after addition of HIgG. Because, HIgG has immunointeraction with anti-HIgG on electrode surface so electrode surface and current signal are decreasing, respectively.

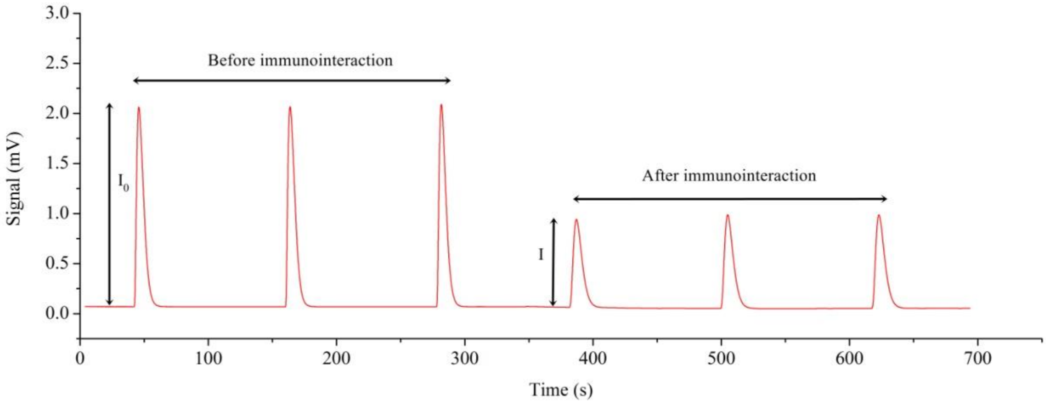

Figure 1.5 SIAgram before and after immunointeraction with HIgG.

The relative decreasing of electrical current was evaluated as:

$$\%\ \text{decreasing current} = \frac{I_0 - I}{I_0} \times 100$$

1.8 Research objectives

The objectives of this research are following:

1.8.1 To develop analytical method that uses sequential injection analysis (SIA) system with amperometric immunosensor to determine human immunoglobulin G (HIgG) by using $[Fe(CN)_6]^{3-/4-}$ as a redox probe

1.8.2 To develop working electrode of an immunosensor that uses screen-printed carbon electrodes (SPCEs) immobilized with graphene oxide (GO) and covalently attached with anti-HIgG

1.8.3 To apply this developed method for real sample analysis such as urine sample